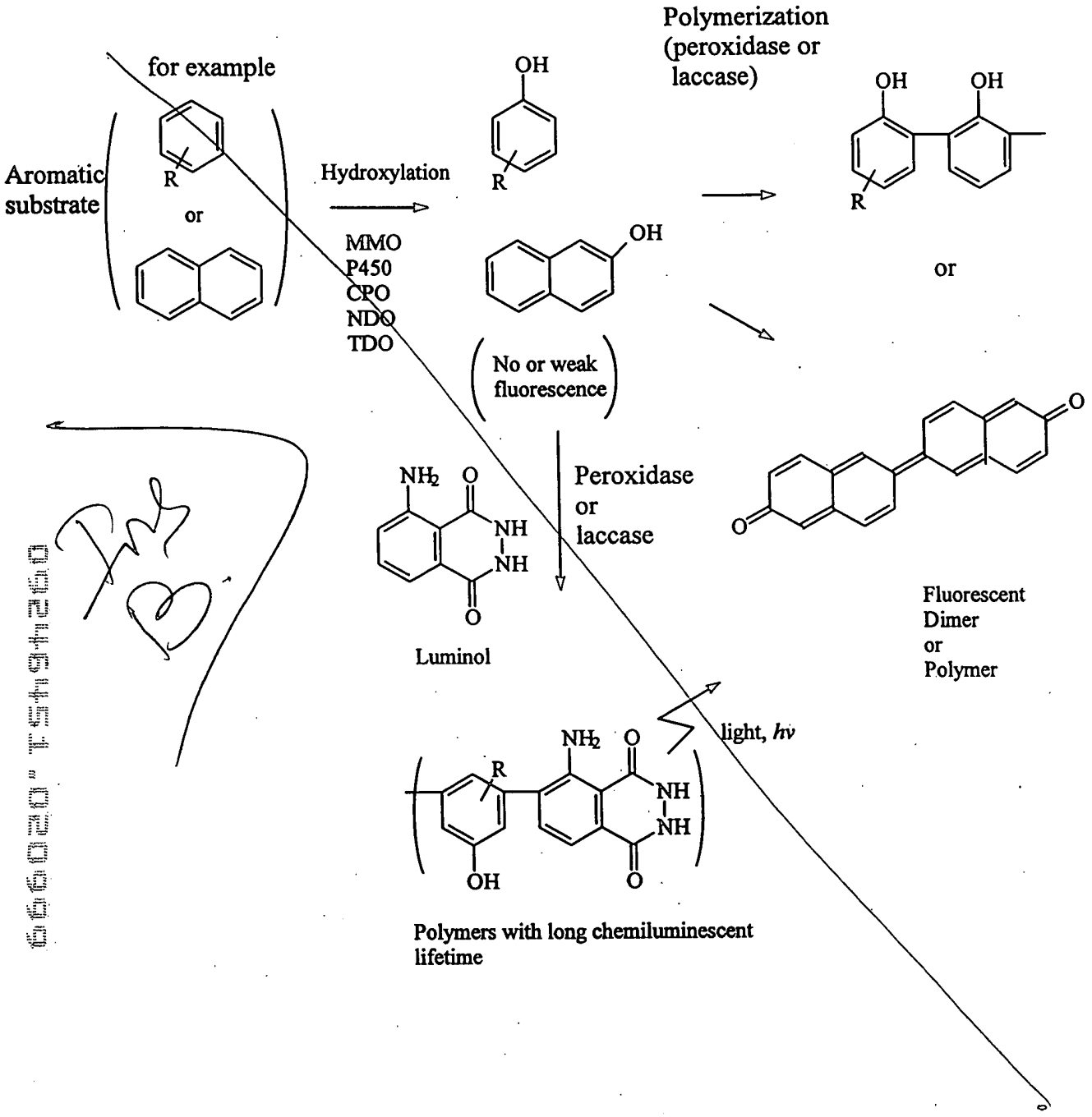

for example
Aromatic substrate
R
or
Hydroxylation
MMO
P450
CPO
NDO
TDO
OH
R
Polymerization (peroxidase or laccase)
OH
OH
R
or
OH
No or weak fluorescence
Peroxidase or laccase
NH2
O
NH
NH
O
Luminol
O
O
Fluorescent Dimer or Polymer
light, hv
NH2
O
R
NH
NH
OH
O
Polymers with long chemiluminescent lifetime

Fig. 1

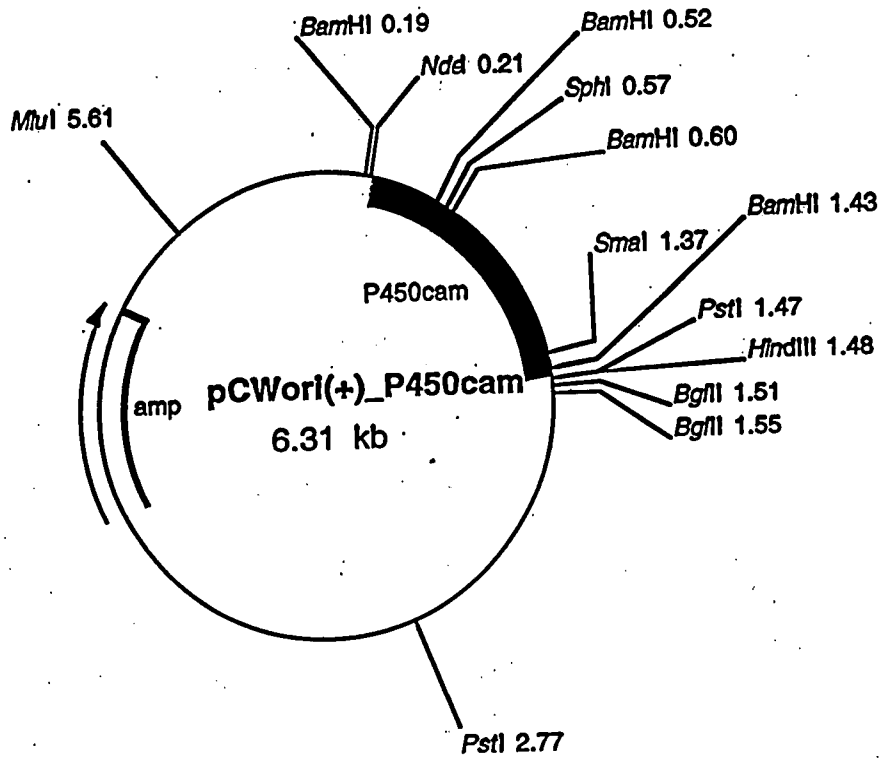


Fig. 2 P450cam dans pCWori(+) graphic map (This vector was constructed and donated by Prof.Ortiz de Montellano).
DNA sequence : 6313 bp. First P*tac* : start at 22 end at 49,
Second P*tac* : start at 117 end at 144

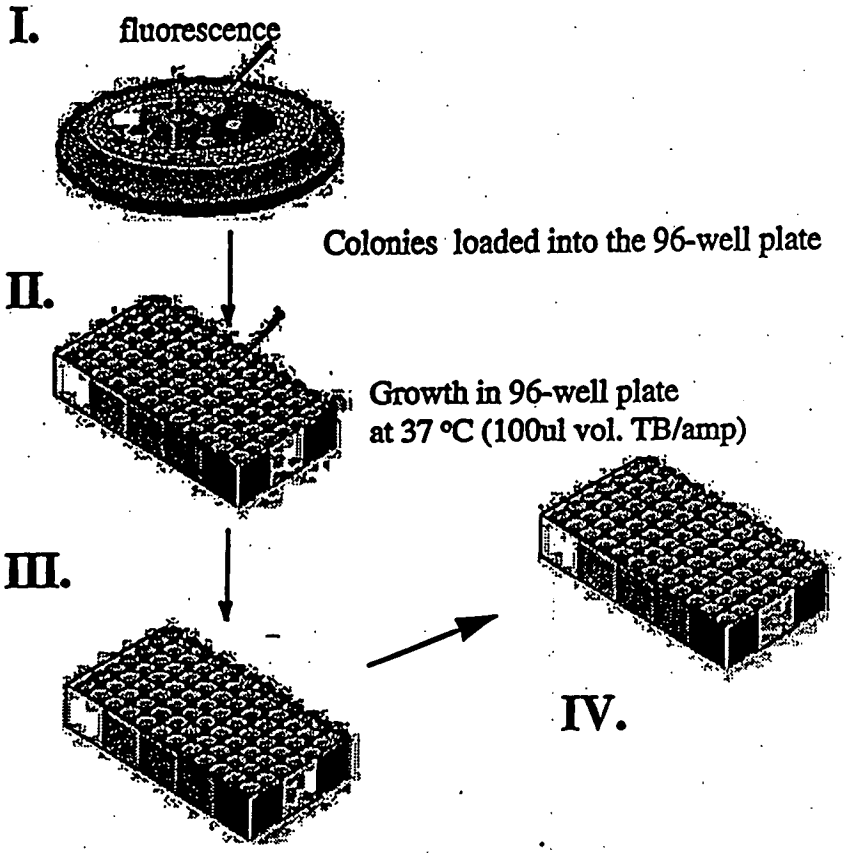
ORIGIN   5 bp upstream of PstI site.

```
   1 CTGCAGGATC GTTATCCGCT GGCCGATCTG ATCACCCAGC GTTTTTCCAT CGACGAGGCC
  61 AGCAAGGCAC TTGAACTGGT CAAGGCAGGA GCACTGATCA AACCCGTGAT CGACTCCACT
 121 CTTTAGCCAA CCCGCGTTCC AGGAGAACAA CAACAATGAC GACTGAAACC ATACAAAGCA
 181 ACGCCAATCT TGCCCCTCTG CCACCCCATG TGCCAGAGCA CCTGGTATTC GACTTCGACA
 241 TGTACAATCC GTCGAATCTG TCTGCCGGCG TGCAGGAGGC CTGGGCAGTT CTGCAAGAAT
 301 CAAACGTACC GGATCTGGTG TGGACTCGCT GCAACGGCGG ACACTGGATC GCCACTCGCG
 361 GCCAACTGAT CCGTGAGGCC TATGAAGATT ACCGCCACTT TTCCAGCGAG TGCCCGTTCA
 421 TCCCTCGTGA AGCCGGCGAA GCCTACGACT TCATTCCCAC CTCGATGGAT CCGCCCGAGC
 481 AGCGCCAGTT TCGTGCGCTG GCCAACCAAG TGGTTGGCAT GCCGGTGGTG GATAAGCTGG
 541 AGAACCGGAT CCAGGAGCTG GCCTGCTCGC TGATCGAGAG CCTGCGCCCG CAAGGACAGT
 601 GCAACTTCAC CGAGGACTAC GCCGAACCCT TCCCGATACG CATCTTCATG CTGCTCGCAG
 661 GTCTACCGGA AGAAGATATC CCGCACTTGA AATACCTAAC GGATCAGATG ACCCGTCCGG
 721 ATGGCAGCAT GACCTTCGCA GAGGCCAAGG AGGCGCTCTA CGACTATCTG ATACCGATCA
 781 TCGAGCAACG CAGGCAGAAG CCGGGAACCG ACGCTATCAG CATCGTTGCC AACGGCCAGG
 841 TCAATGGGCG ACCGATCACC AGTGACGAAG CCAAGAGGAT GTGTGGCCTG TTACTGGTCG
 901 GCGGCCTGGA TACGGTGGTC AATTTCCTCA GCTTCAGCAT GGAGTTCCTG GCCAAAAGCC
 961 CGGAGCATCG CCAGGAGCTG ATCGAGCGTC CCGAGCGTAT TCCAGCCGCT TGCGAGGAAC
1021 TACTCCGGCG CTTCTCGCTG GTTGCCGATG GCCGCATCCT CACCTCCGAT TACGAGTTTC
1081 ATGGCGTGCA ACTGAAGAAA GGTGACCAGA TCCTGCTACC GCAGATGCTG TCTGGCCTGG
1141 ATGAGCGCGA AAACGCCTGC CCGATGCACG TCGACTTCAG TCGCCAAAAG GTTTCACACA
1201 CCACCTTTGG CCACGGCAGC CATCTGTGCC TTGGCCAGCA CCTGGCCCGC CGGGAAATCA
1261 TCGTCACCCT CAAGGAATGG CTGACCAGGA TTCCTGACTT CTCCATTGCC CCGGGTGCCC
1321 AGATTCAGCA CAAGAGCGGC ATCGTCAGCG GCGTGCAGGC ACTCCCTCTG GTCTGGGATC
1381 CGGCGACTAC CAAAGCGGTA TA
```

Fig. 3A P450cam ORF Region.

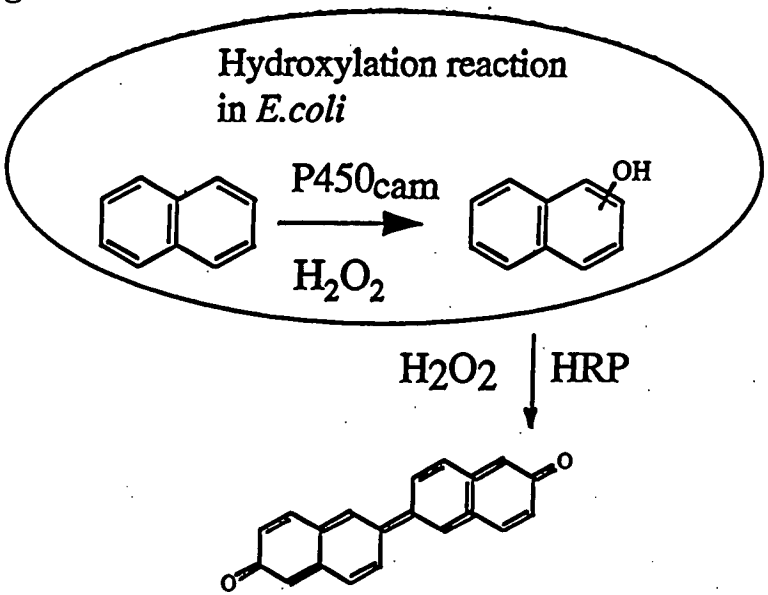
Fig. 3B P450cam amino acid sequence

THR THR GLU THR ILE GLN SER ASN ALA ASN LEU ALA PRO
LEU PRO PRO HIS VAL PRO GLU HIS LEU VAL PHE ASP PHE
ASP MET TYR ASN PRO SER ASN LEU SER ALA GLY VAL GLN
GLU ALA TRP ALA VAL LEU GLN GLU SER ASN VAL PRO ASP
LEU VAL TRP THR ARG CYS ASN GLY GLY HIS TRP ILE ALA
THR ARG GLY GLN LEU ILE ARG GLU ALA TYR GLU ASP TYR
ARG HIS PHE SER SER GLU CYS PRO PHE ILE PRO ARG GLU
ALA GLY GLU ALA TYR ASP PHE ILE PRO THR SER MET ASP
PRO PRO GLU GLN ARG GLN PHE ARG ALA LEU ALA ASN GLN
VAL VAL GLY MET PRO VAL VAL ASP LYS LEU GLU ASN ARG
ILE GLN GLU LEU ALA CYS SER LEU ILE GLU SER LEU ARG
PRO GLN GLY GLN CYS ASN PHE THR GLU ASP TYR ALA GLU
PRO PHE PRO ILE ARG ILE PHE MET LEU LEU ALA GLY LEU
PRO GLU GLU ASP ILE PRO HIS LEU LYS TYR LEU THR ASP
GLN MET THR ARG PRO ASP GLY SER MET THR PHE ALA GLU
ALA LYS GLU ALA LEU TYR ASP TYR LEU ILE PRO ILE ILE
GLU GLN ARG ARG GLN LYS PRO GLY THR ASP ALA ILE SER
ILE VAL ALA ASN GLY GLN VAL ASN GLY ARG PRO ILE THR
SER ASP GLU ALA LYS ARG MET CYS GLY LEU LEU LEU VAL
GLY GLY LEU ASP THR VAL VAL ASN PHE LEU SER PHE SER
MET GLU PHE LEU ALA LYS SER PRO GLU HIS ARG GLN GLU
LEU ILE GLU ARG PRO GLU ARG ILE PRO ALA ALA CYS GLU
GLU LEU LEU ARG ARG PHE SER LEU VAL ALA ASP GLY ARG
ILE LEU THR SER ASP TYR GLU PHE HIS GLY VAL GLN LEU
LYS LYS GLY ASP GLN ILE LEU LEU PRO GLN MET LEU SER
GLY LEU ASP GLU ARG GLU ASN ALA CYS PRO MET HIS VAL
ASP PHE SER ARG GLN LYS VAL SER HIS THR THR PHE GLY
HIS GLY SER HIS LEU CYS LEU GLY GLN HIS LEU ALA ARG
ARG GLU ILE ILE VAL THR LEU LYS GLU TRP LEU THR ARG
ILE PRO ASP PHE SER ILE ALA PRO GLY ALA GLN ILE GLN
HIS LYS SER GLY ILE VAL SER GLY VAL GLN ALA LEU PRO
LEU VAL TRP ASP PRO ALA THR THR LYS ALA VAL



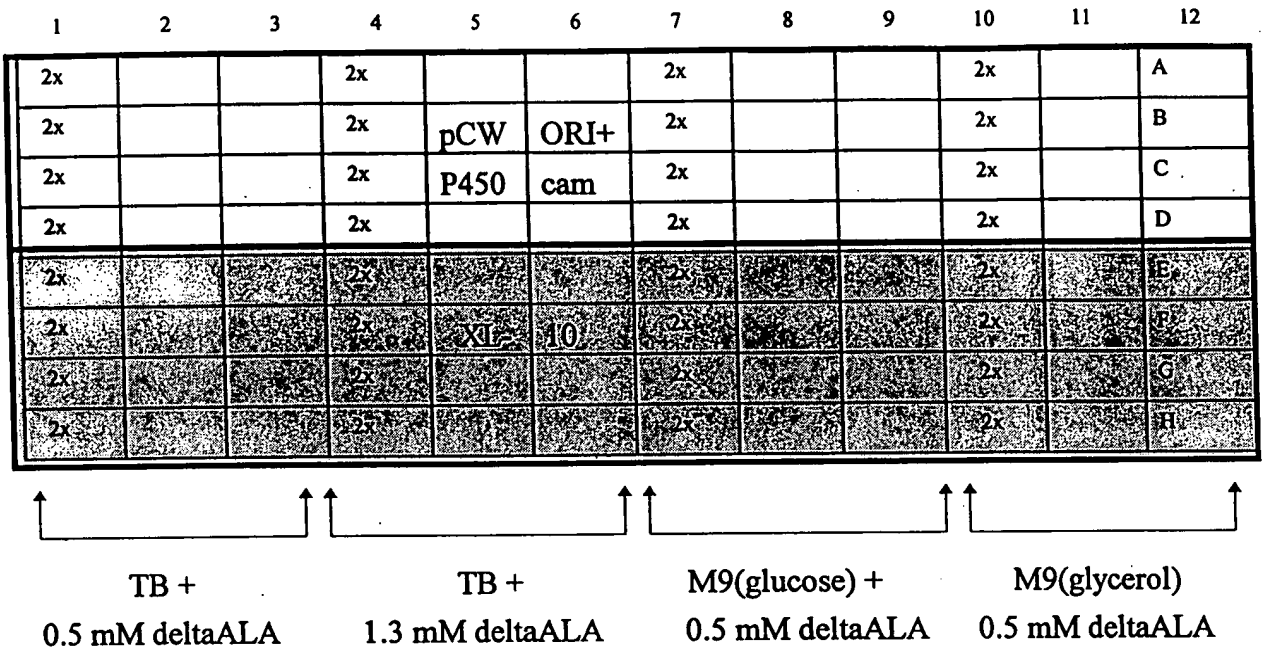
$P450_{cam}$ protein expression by adding 0.5 -1 mM IPTG
and 1 mM thiamine (XL-10), 0.5-1.3 mM delta-ALA,
0.5ml trace element stock/10ml medium (total: 120ul. volume)
Induction time : 24 hr
Induction temperature : 30 °C

Fig. 4B



Reaction mixture (total 200 ul vol):
- 50 ul. culure broth
- 100 ul. sodium dibasic phosphate buffer (50 mM, pH9)
- 10 ul. pure ethanol
- 20 ul. naphthalene stock(1g/13 ml in pure ethanol).
- 10 ul. hydrogen peroxide stock solution (100 mM)
- 10 ul. horseradish peroxidase stock solution
(1400units/10 ml)

Fig. 4A $P450_{cam}$ activity assay using added horseradish peroxidase (HRP).



* 2x : 200 μ L cultivation volume, others : 100 μ L cultivation volume.

Fig. 5A

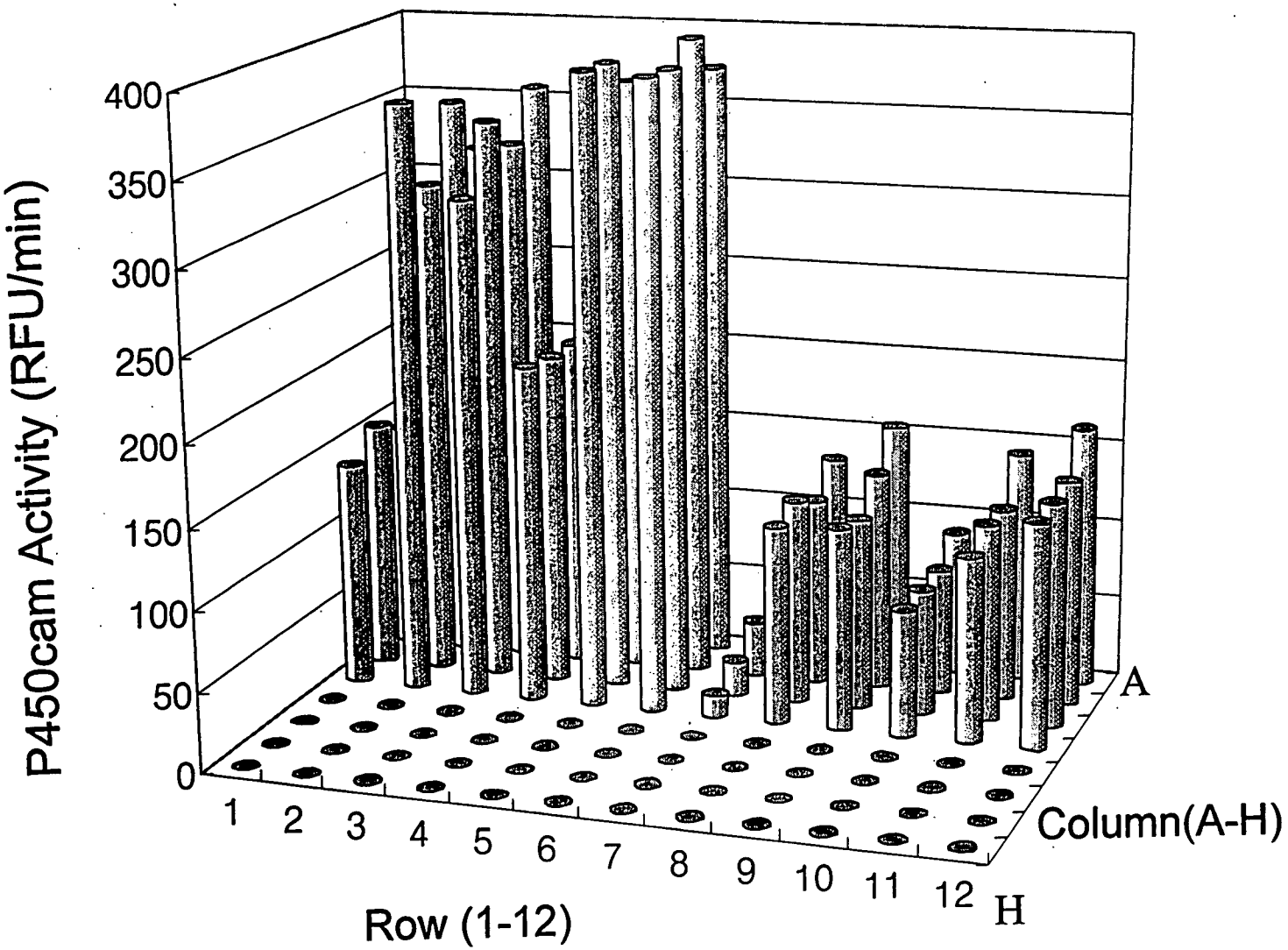

P450cam Activity (RFU/min)
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
Row (1-12)
A
H
Column(A-H)

Fig. 5B

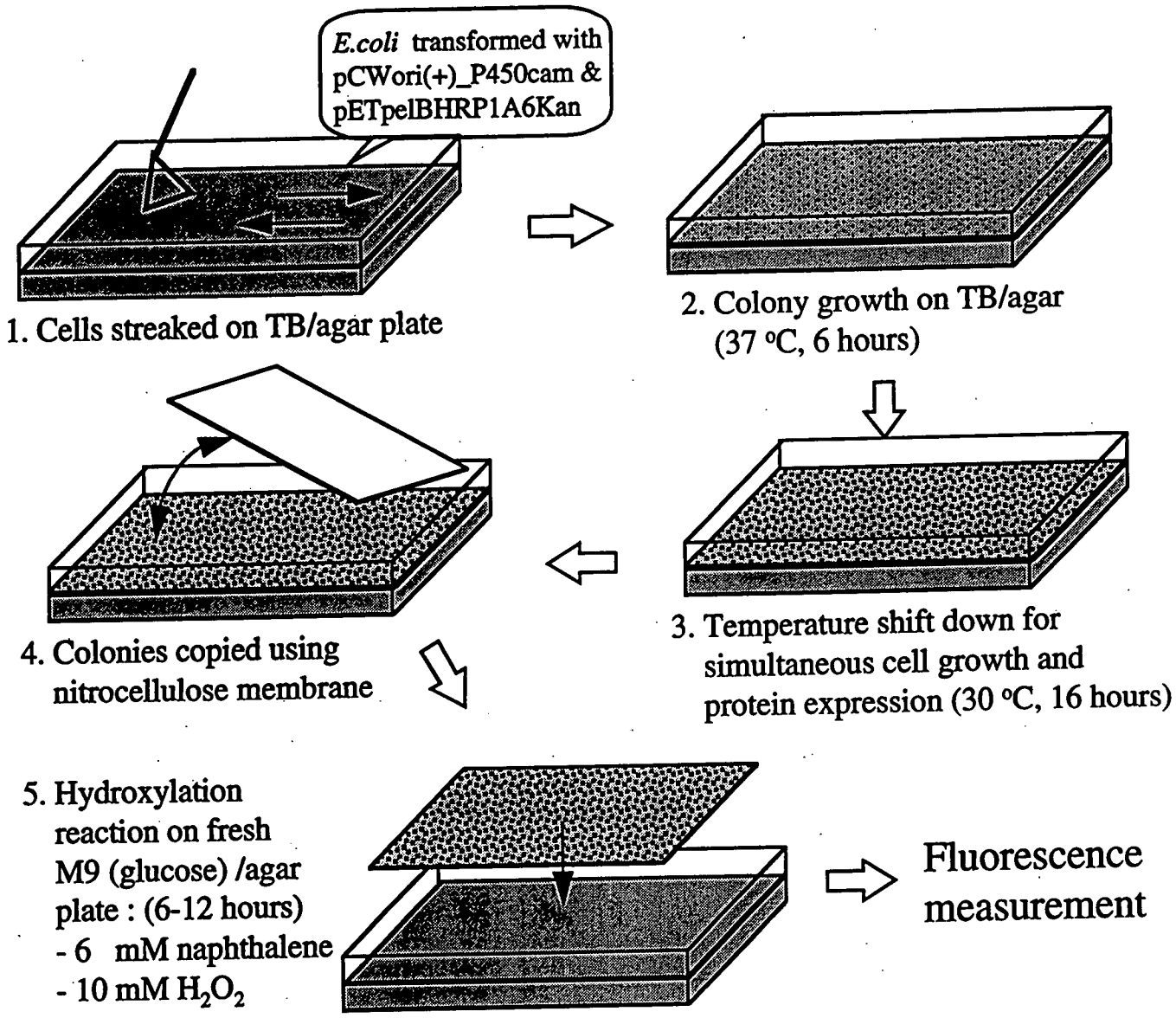


Fig. 6

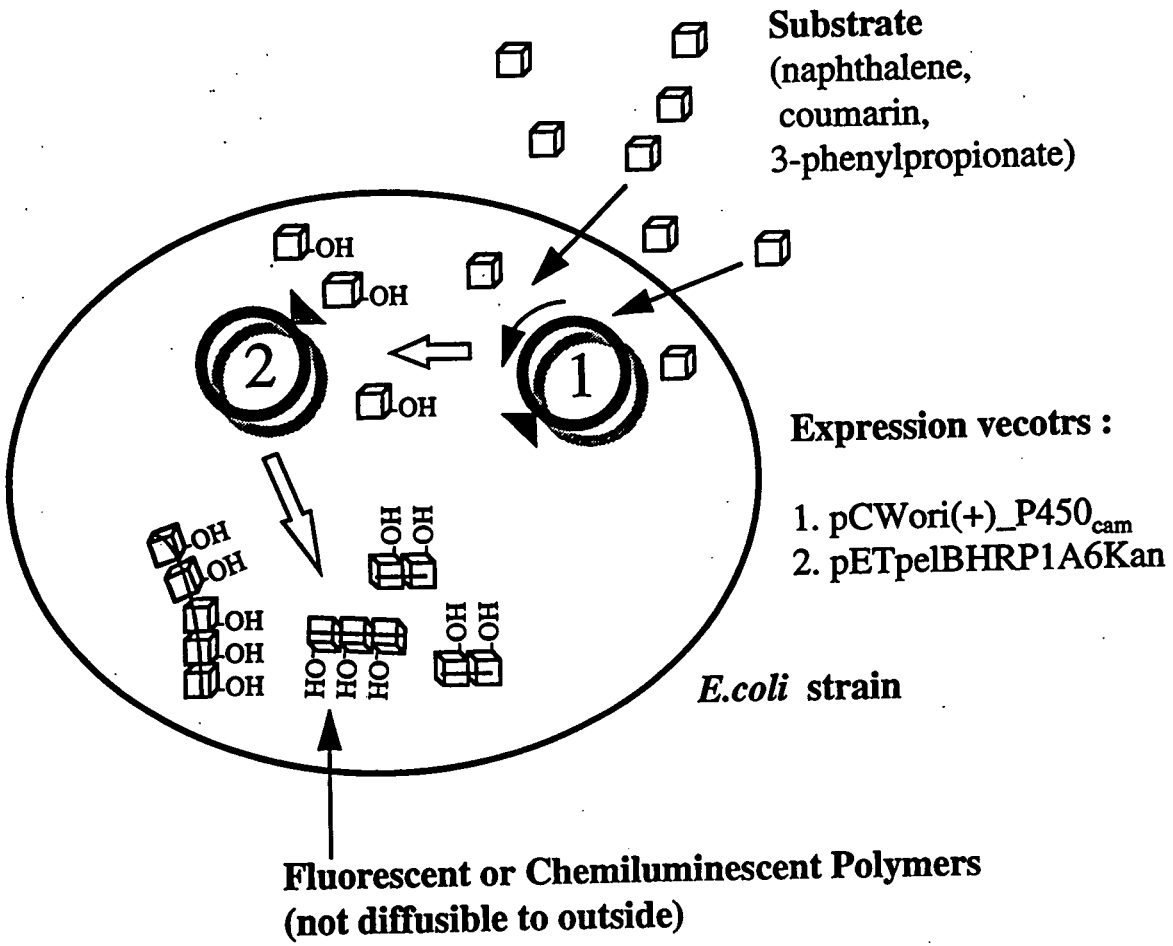


Fig. 7

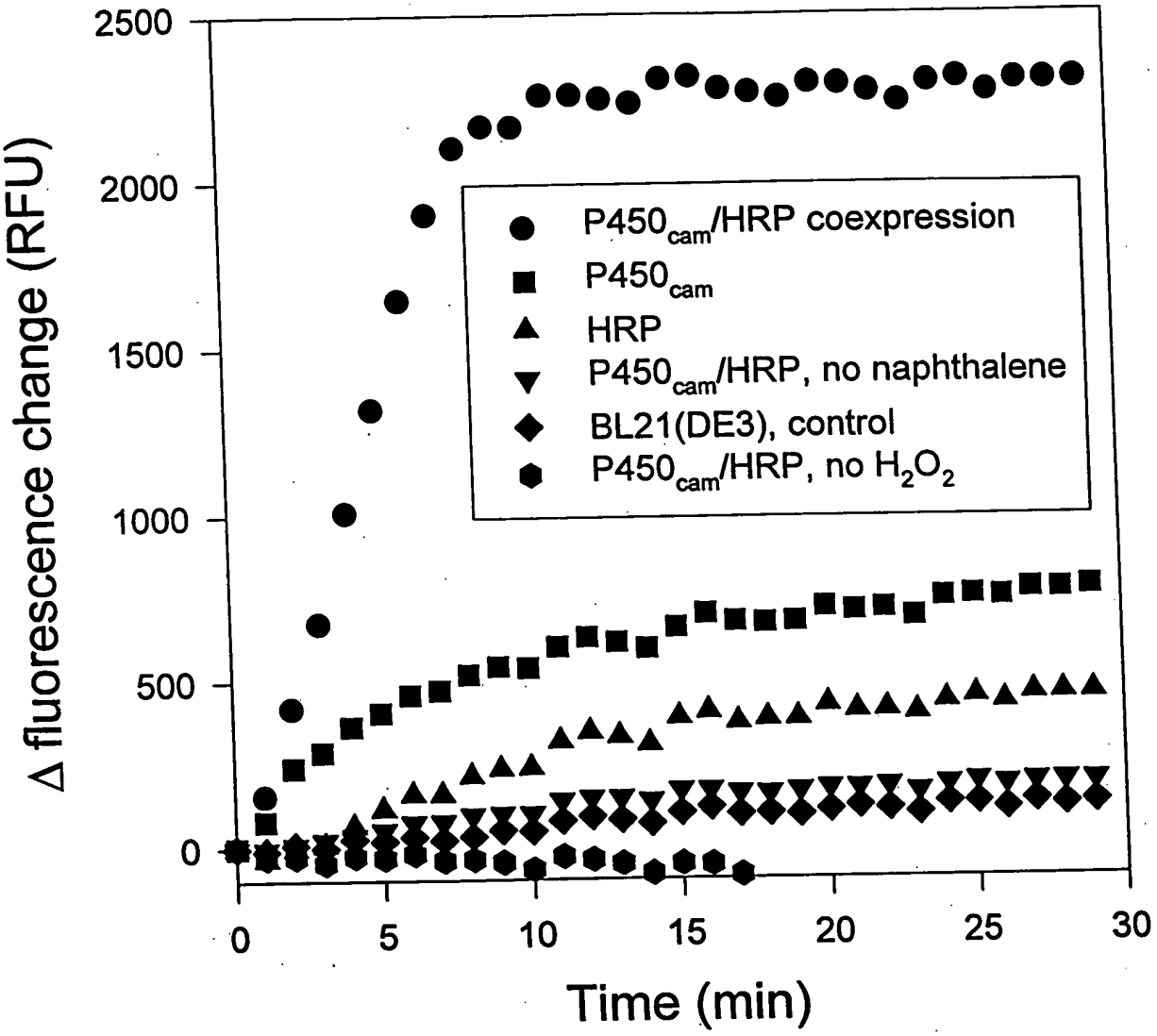

2500
2000
1500
1000
500
0
Δ fluorescence change (RFU)
0
5
10
15
20
25
30
Time (min)
P450cam/HRP coexpression
P450cam
HRP
P450cam/HRP, no naphthalene
BL21(DE3), control
P450cam/HRP, no H2O2

Fig. 8

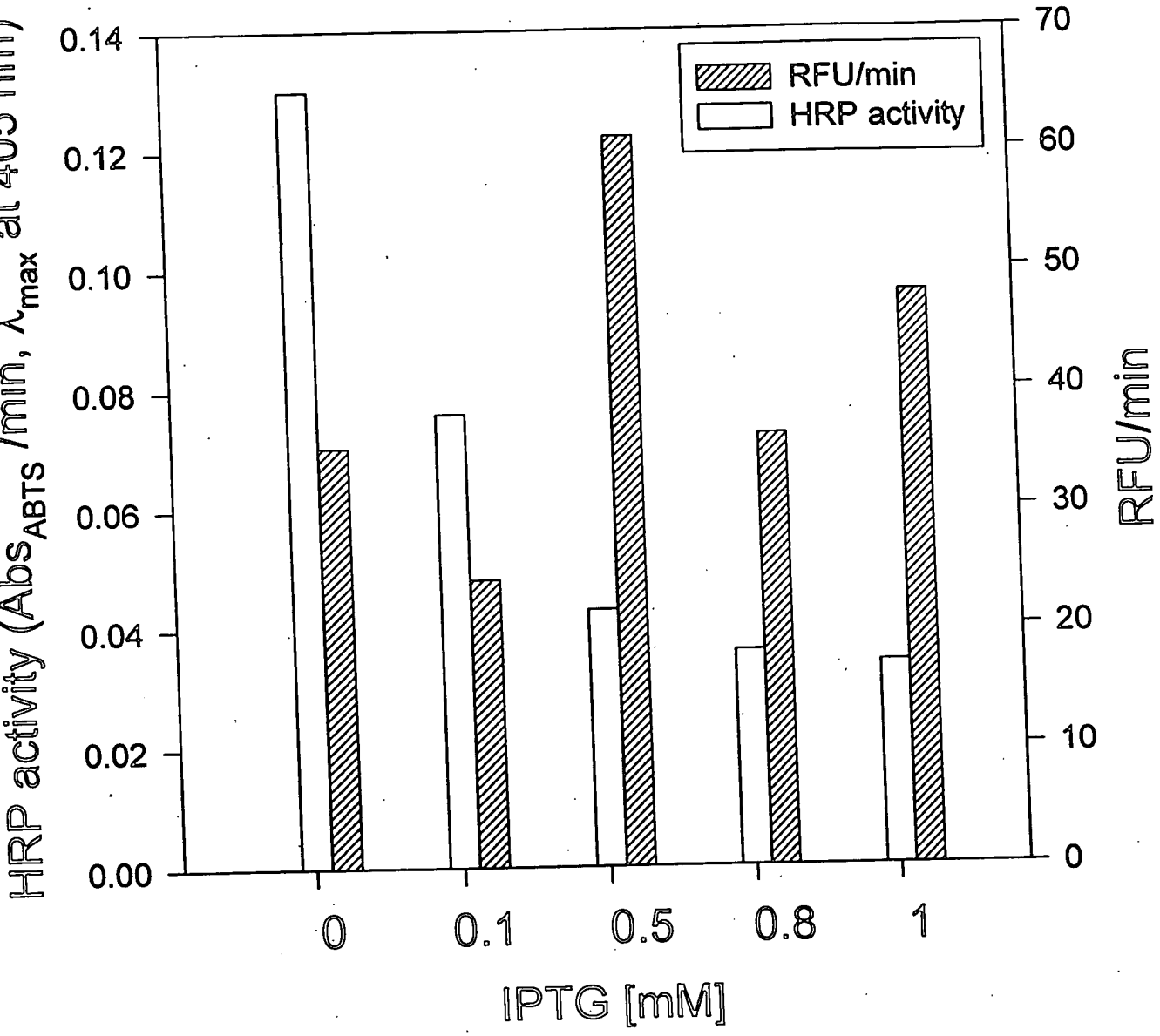
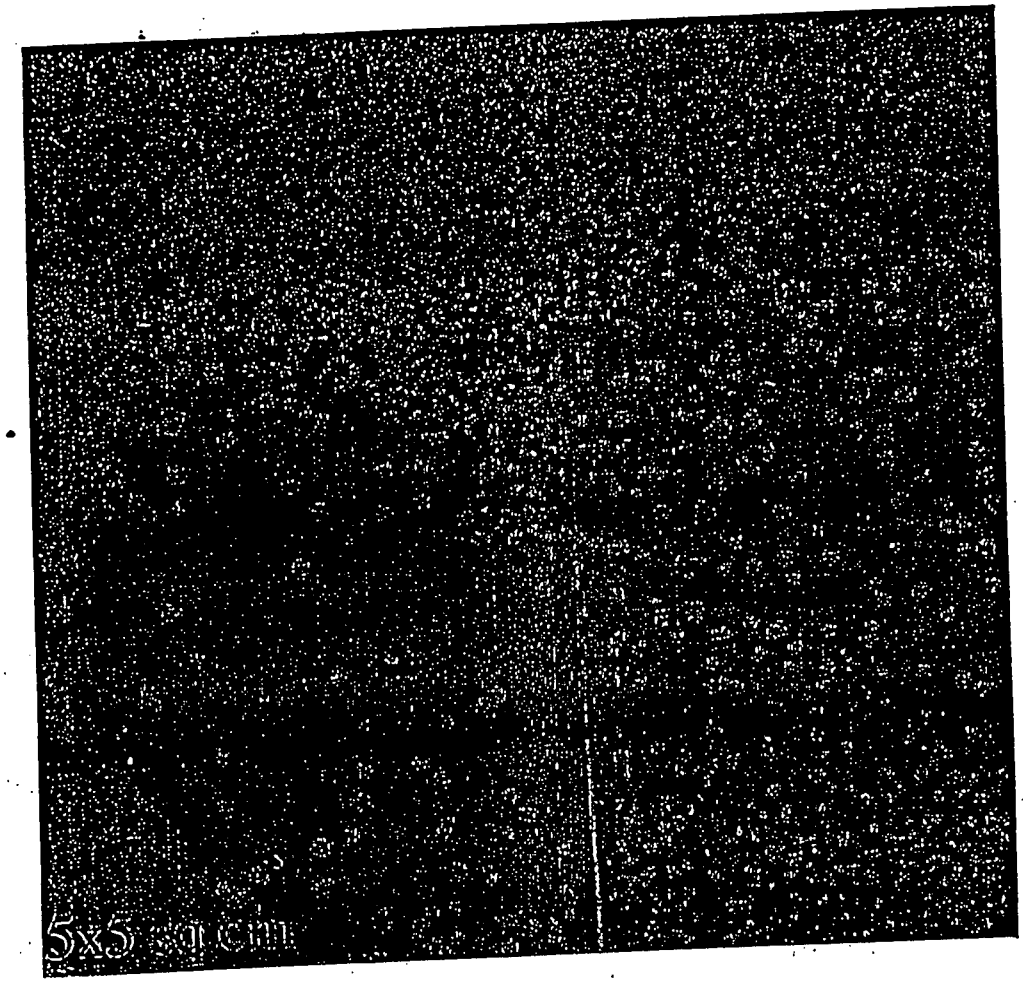

RFU/min
HRP activity
HRP activity (Abs$_{ABTS}$ /min, λ_{max} at 405 nm)
RFU/min
IPTG [mM]
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
70
60
50
40
30
20
10
0
0
0.1
0.5
0.8
1

Fig. 9



E. Coli BL-21(DE3) showing strong intracellular fluorescence in the presence naphthalene and hydrogen peroxide: $P450_{cam}$ and *mutant* HRP were coexpressed in the cells. Hydroxylated naphthols generated by P450cam reaction was intensified by the HRP mediated coupling reaction

Fig. 10

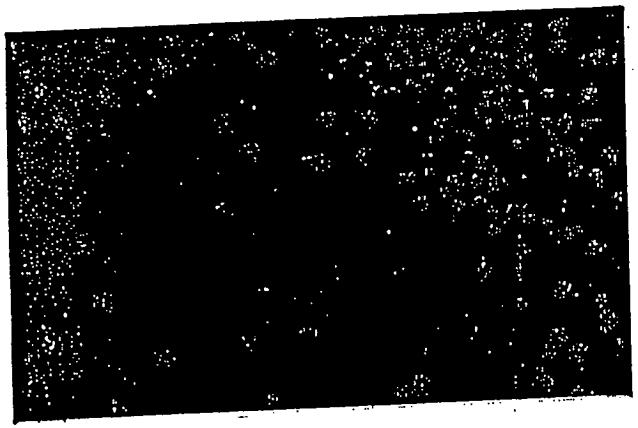


Fig. 11A

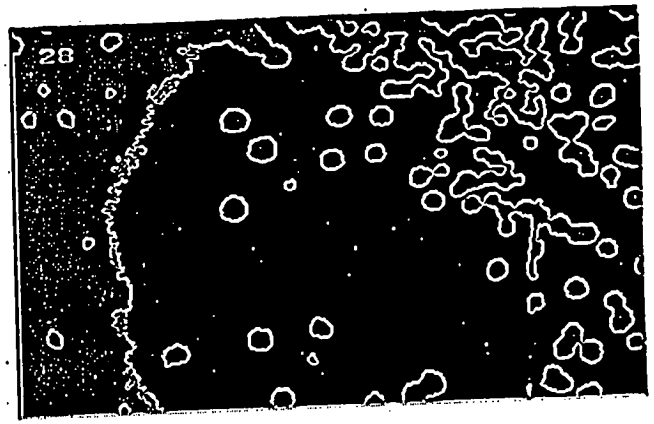


Fig. 11B

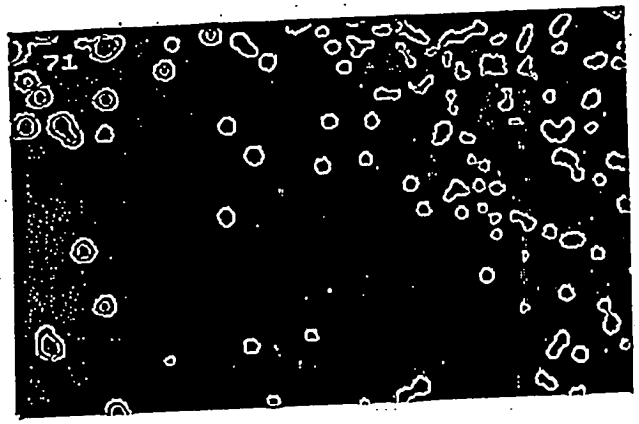


Fig. 11C

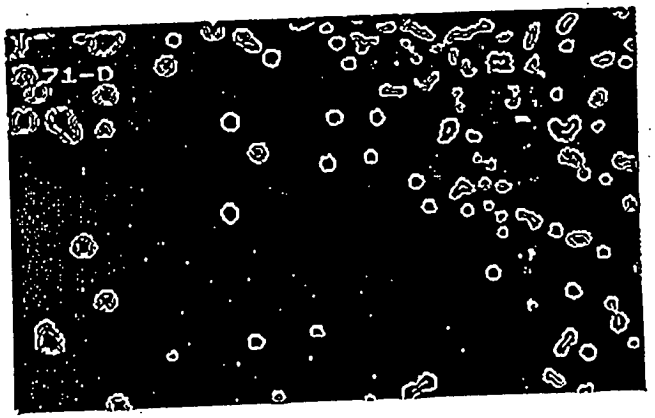


Fig. 11D

a|b|c|d Labeled section, a) original fluorescence image, b) locally improved by cutting touching cells:threshold value = 28, c) cleaned by edge cut and deletion, threshold level = 71, d) finding and dividing overlap region

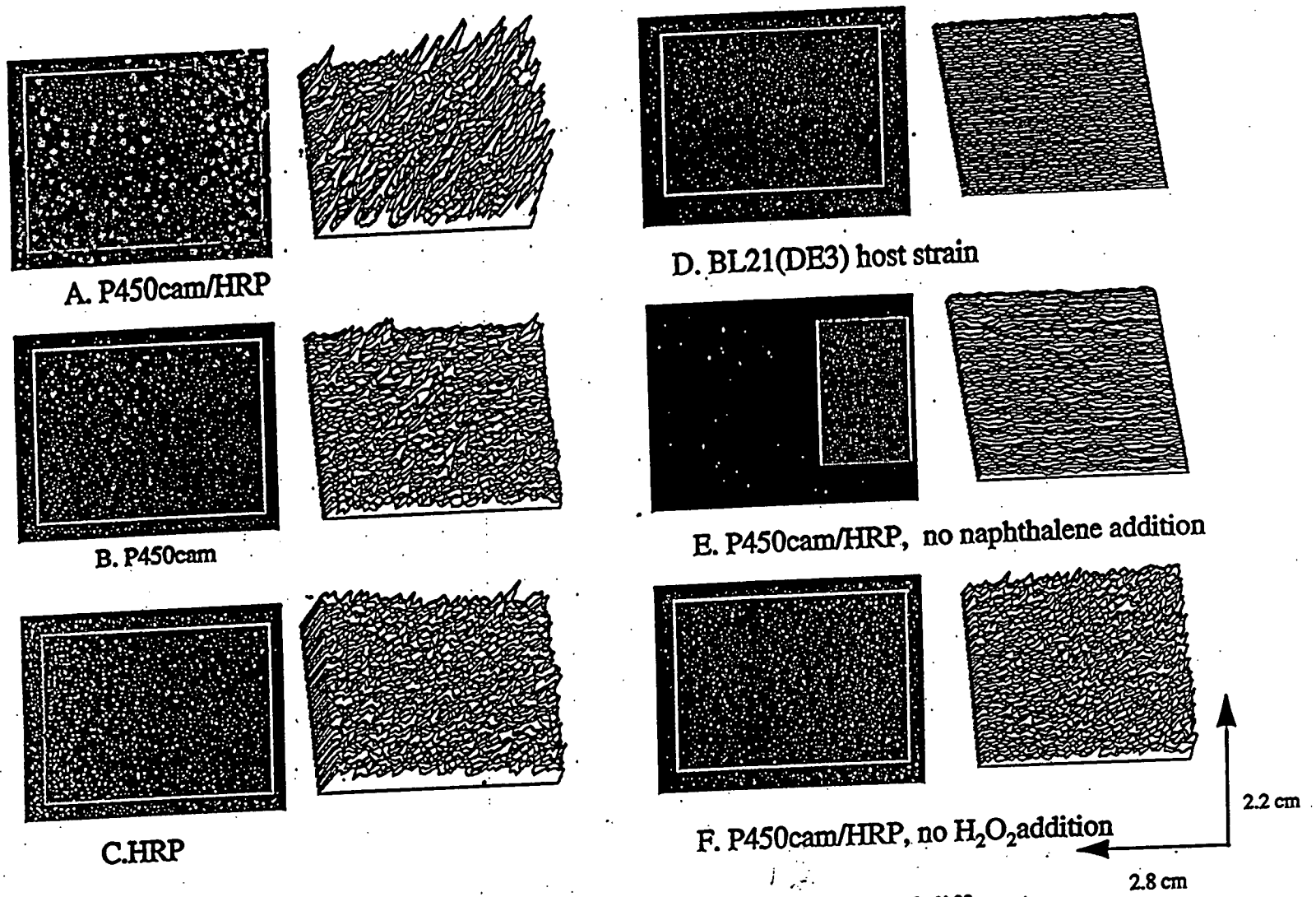


Image analysis result showing fluorescence intensities under 6 different conditions

Fig. 12

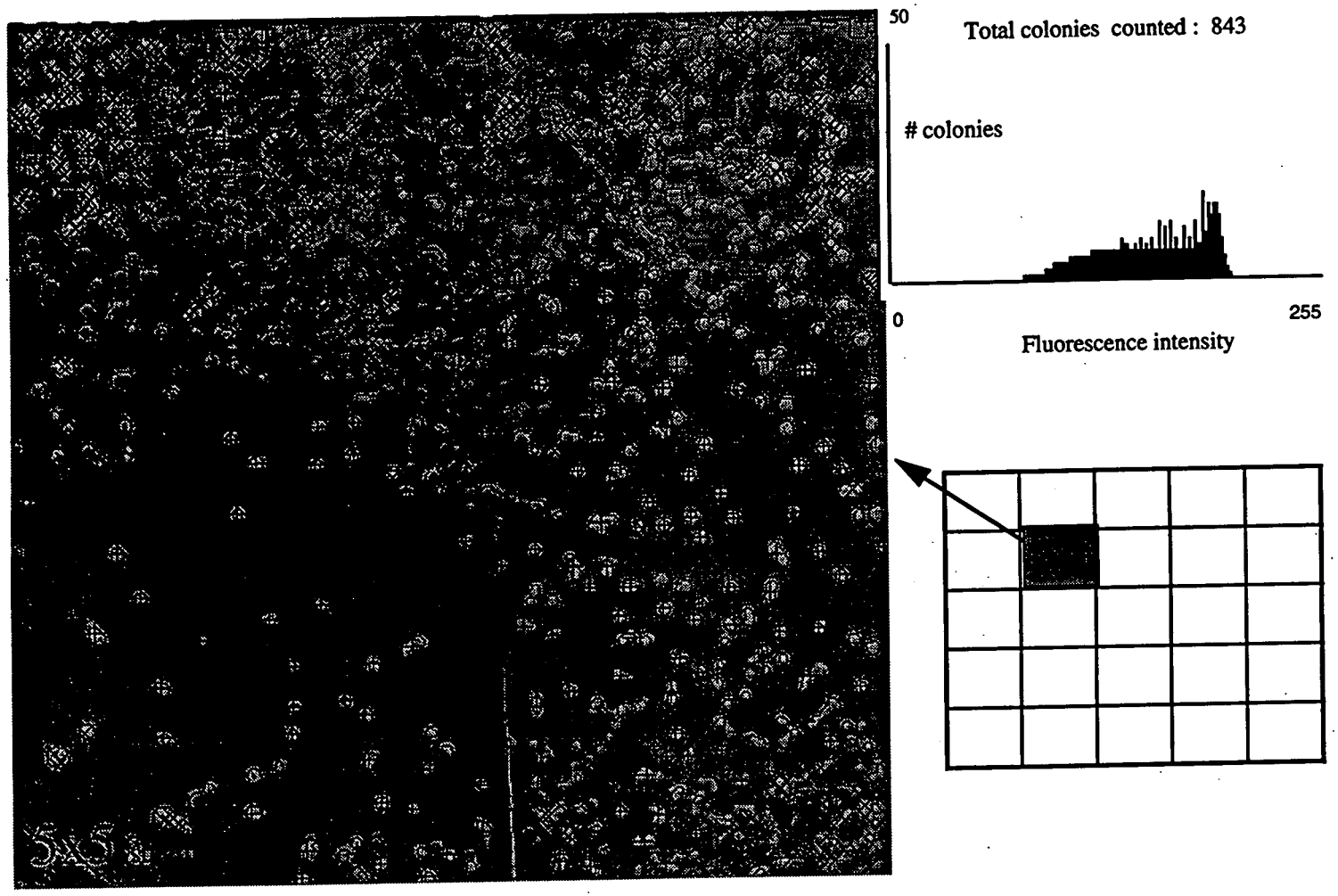
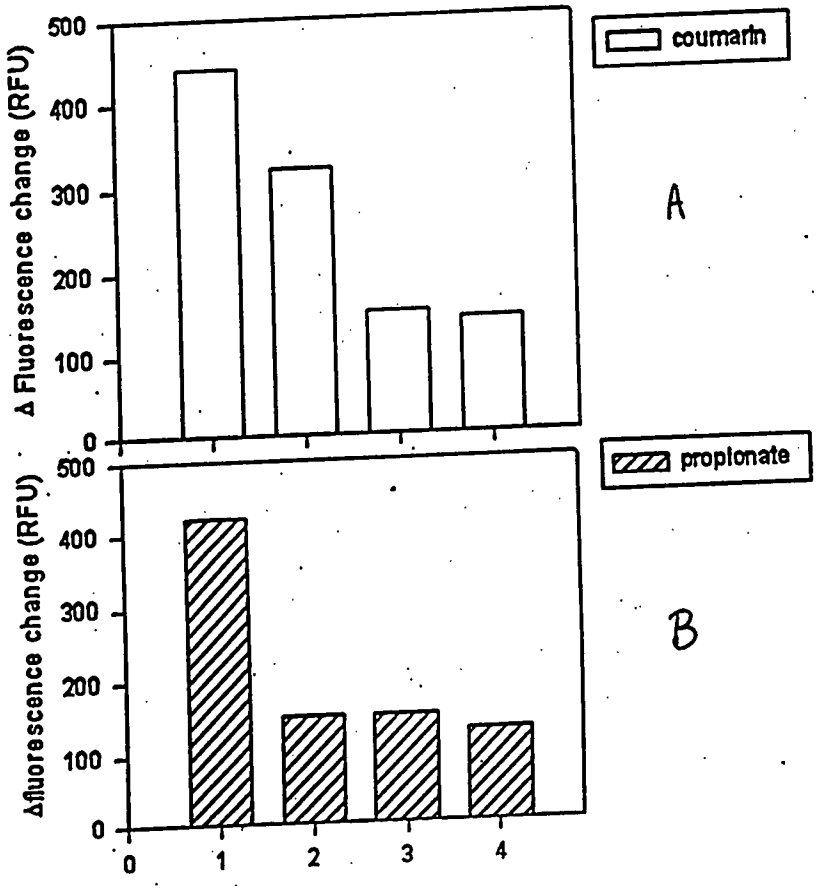
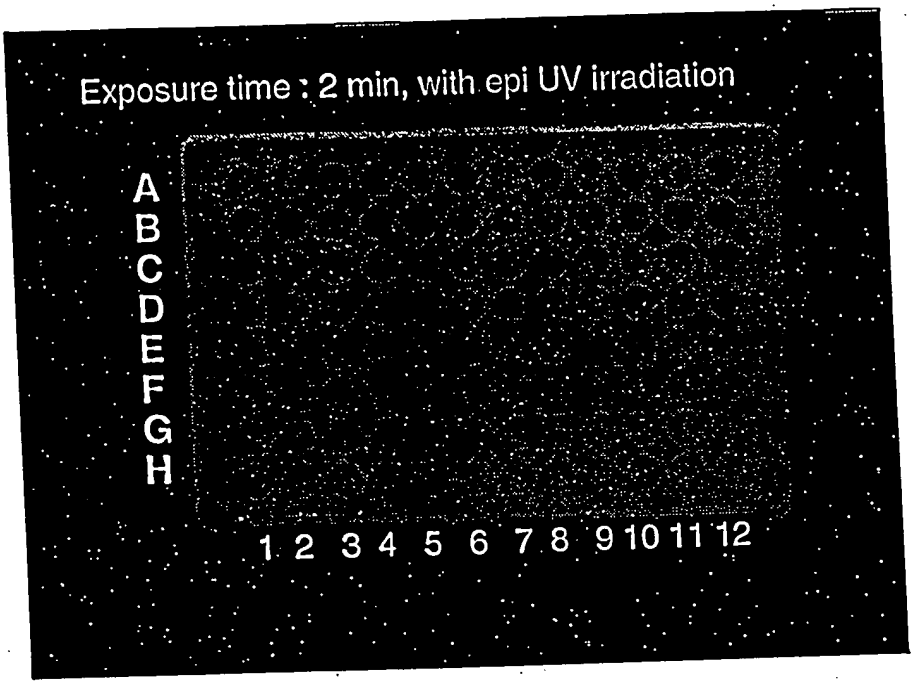

50
Total colonies counted : 843
colonies
0
255
Fluorescence intensity

Fig. 13



Fluorescence Intensification using P450cam/HRP1A6 coexpression system. (1) P450cam/HRP1A6 in BL21(DE3), (2) P450cam in BL2/(DE3), (3) HRP1A6 in BL21(DE3), (4) BL21(DE3) host strain

Fig. 14



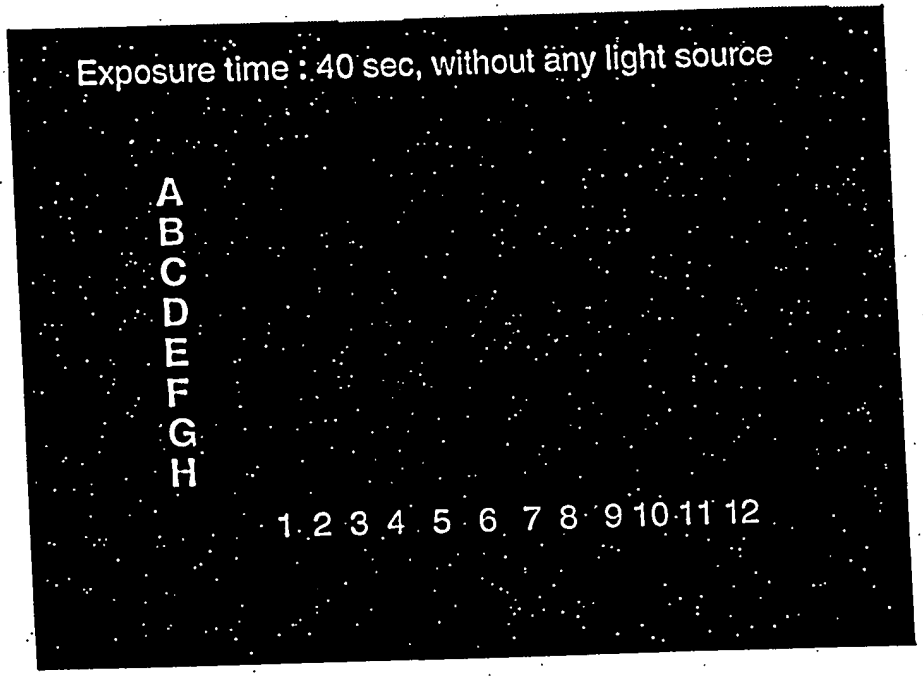
Row :

E : 60 uM luminol + 0.5 mM PPP
F : 120 uM luminol + 0.5 mM PPP
G : 60 uM luminol
H : 120 uM luminol

Column :

4 : P450cam/HRP1A6 in BL21(DE3)
5 : P450cam in BL21(DE3)
6 : HRP1A6 in BL21(DE3)
7 : host strain, BL21(DE3)

Fig. 15A



Light Emission values

E4 : 51 ILDV
F4 : 98 ILDV
G4 : 0.2 ILDV
H4 : 1 ILDV

Others : < 0.1 ILDV

Fig. 15B

~~Fig. 4.~~1. The hydroxylation assay using chemiluminescence detection. Hydroxylated *p*-phenyl propionate (catalyzed by P450cam) is utilized in the second, HRP catalyzed reaction with luminol. The light emissions were enhanced up to 98 ILDV (integrated light density value) from < 0.1 background level.

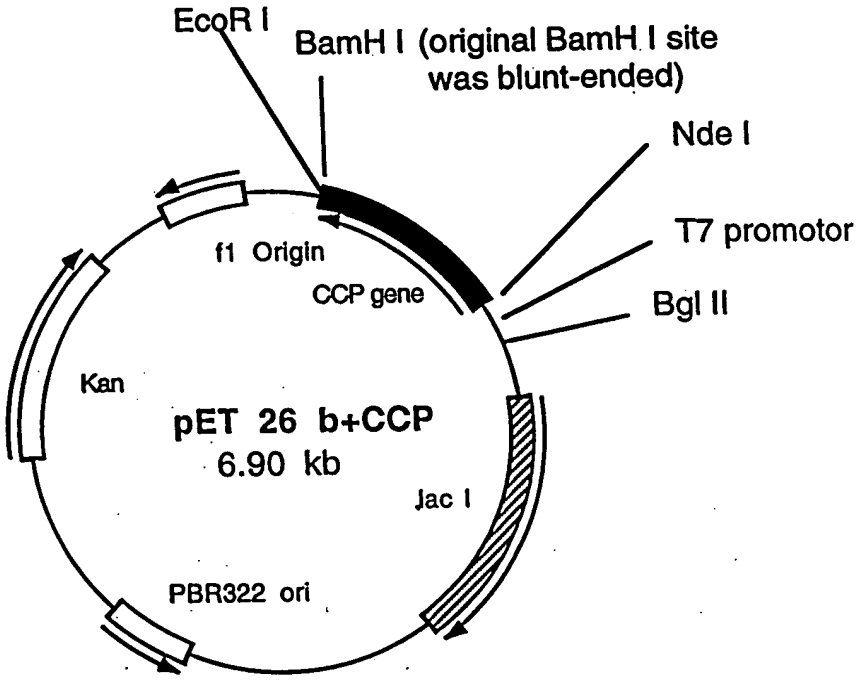


Fig. 16 Yeast cytochrome c peroxidase expression vector pET26 b+CCP. pelB leader sequence in original pET 26 b+ vector was deleted for intracellular CCP expression in *E.coli*.

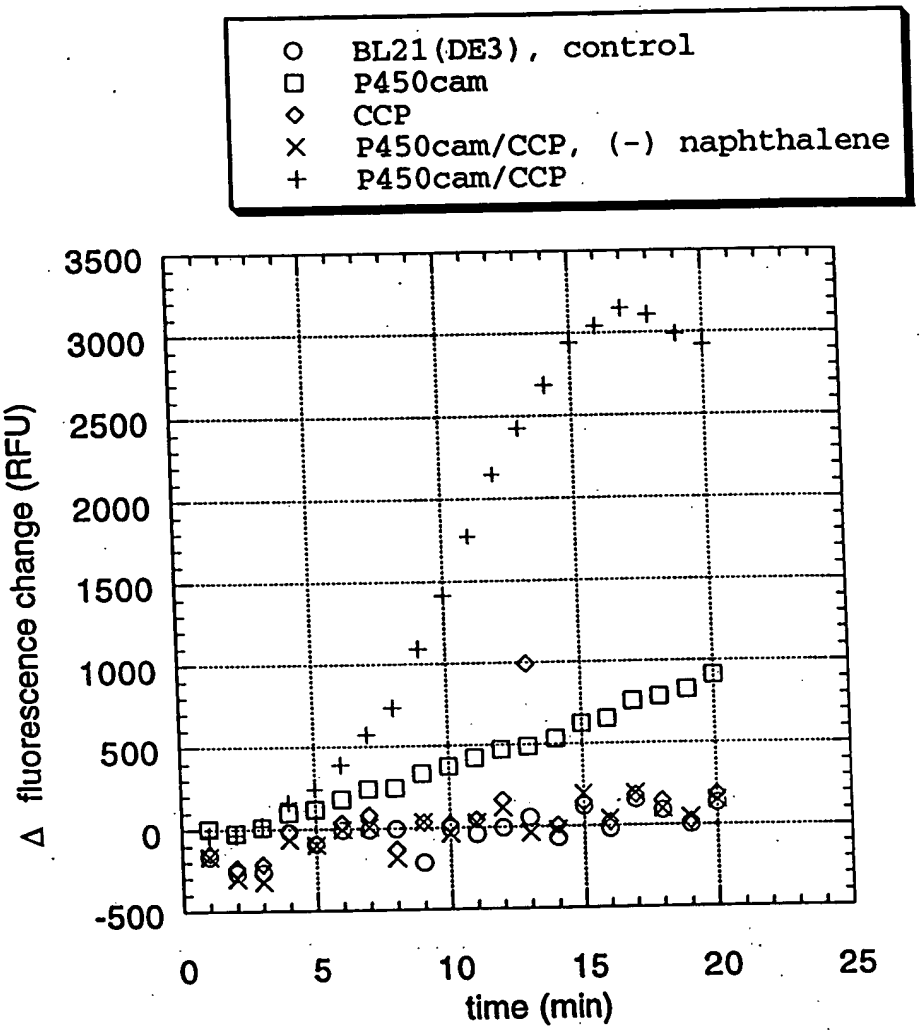


Fig. 17 Development of fluorescence in solutions containing naphthalene and hydrogen peroxide. Reactions were carried out using whole cells and pH 9.0 dibasic sodium phosphate buffer (100mM).

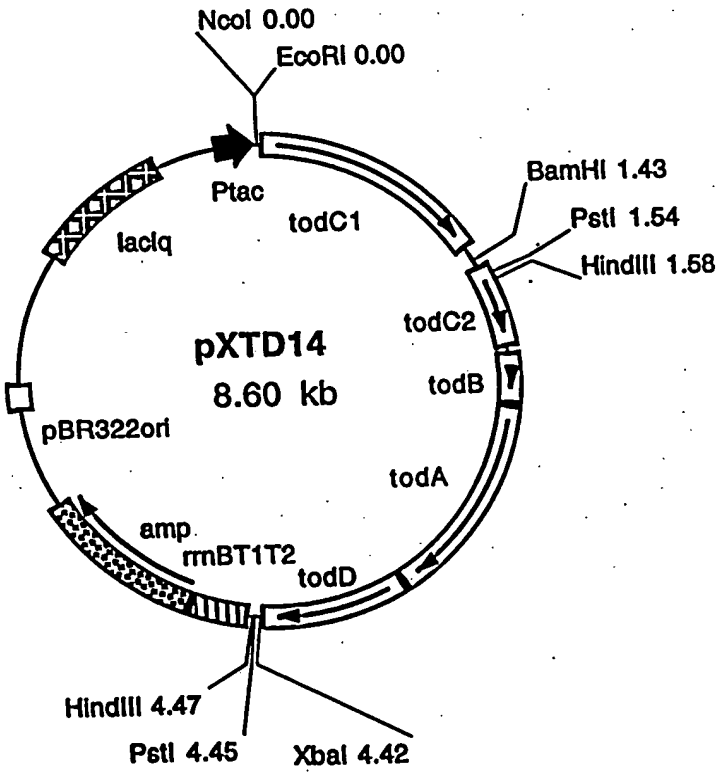

NcoI 0.00
EcoRI 0.00
BamHI 1.43
PstI 1.54
HindIII 1.58
Ptac
todC1
lacIq
todC2
pXTD14
8.60 kb
todB
pBR322ori
todA
amp
rrnBT1T2
todD
HindIII 4.47
PstI 4.45
XbaI 4.42

Fig. 18

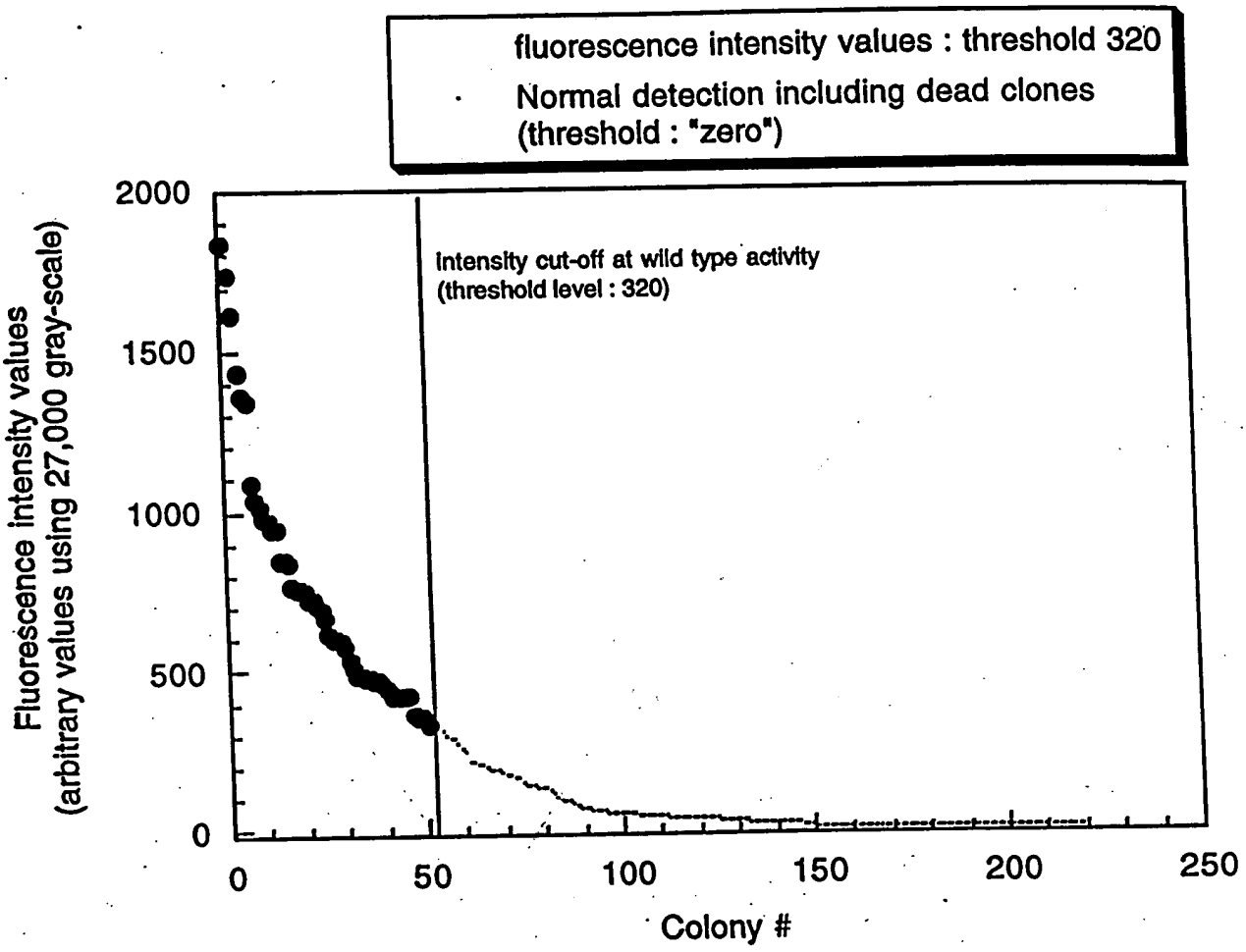


Fig. 19A Activities of P450cam mutants as measured by fluorescence digital imaging.

Fig. 19C

Fig. 19D

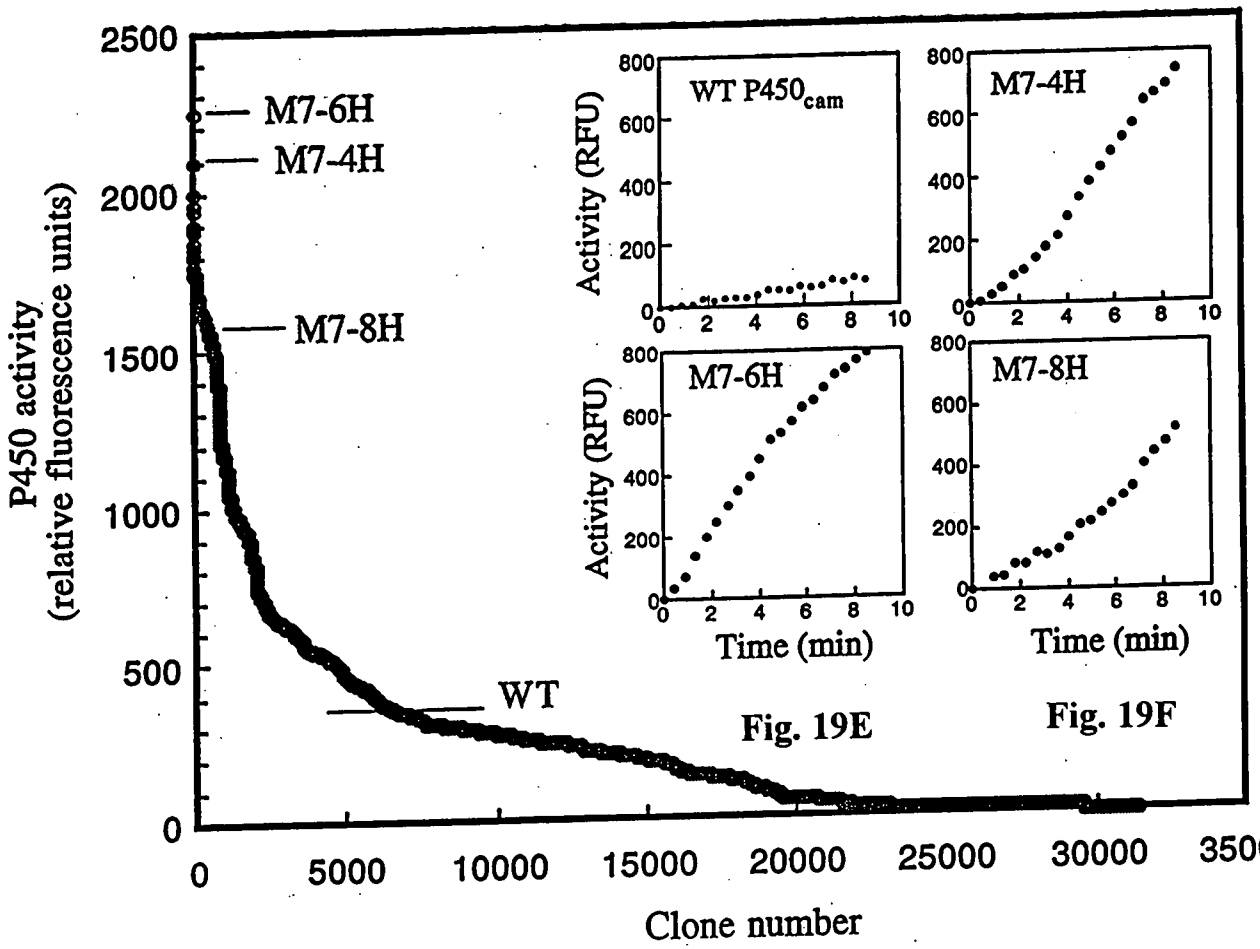


Fig. 19E

Fig. 19F

Fig. 19B

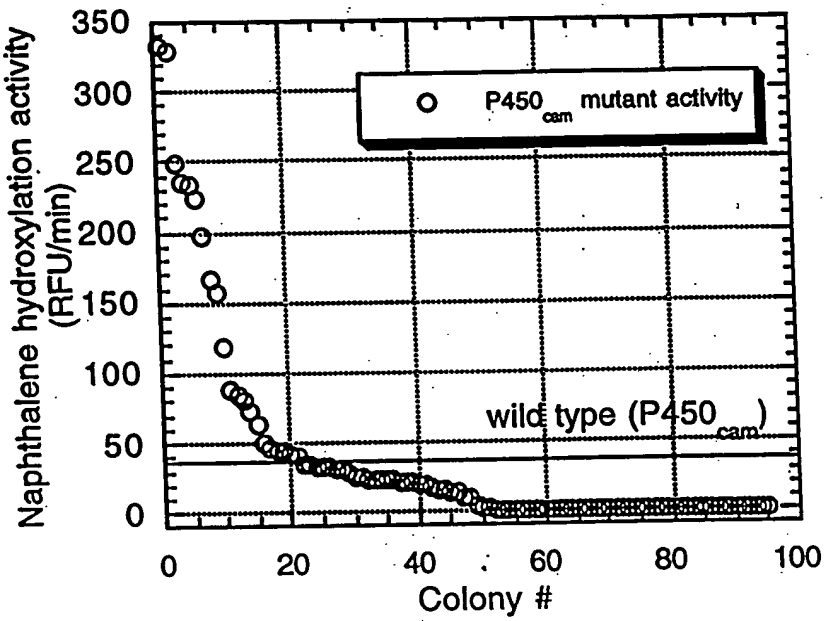


Fig. 20 Activities of randomly chosen P450cam mutants in 96-well plate assay, as measured by fluorescence. Activity of wild type P450cam is approximately 40 RFU/min under these conditions.

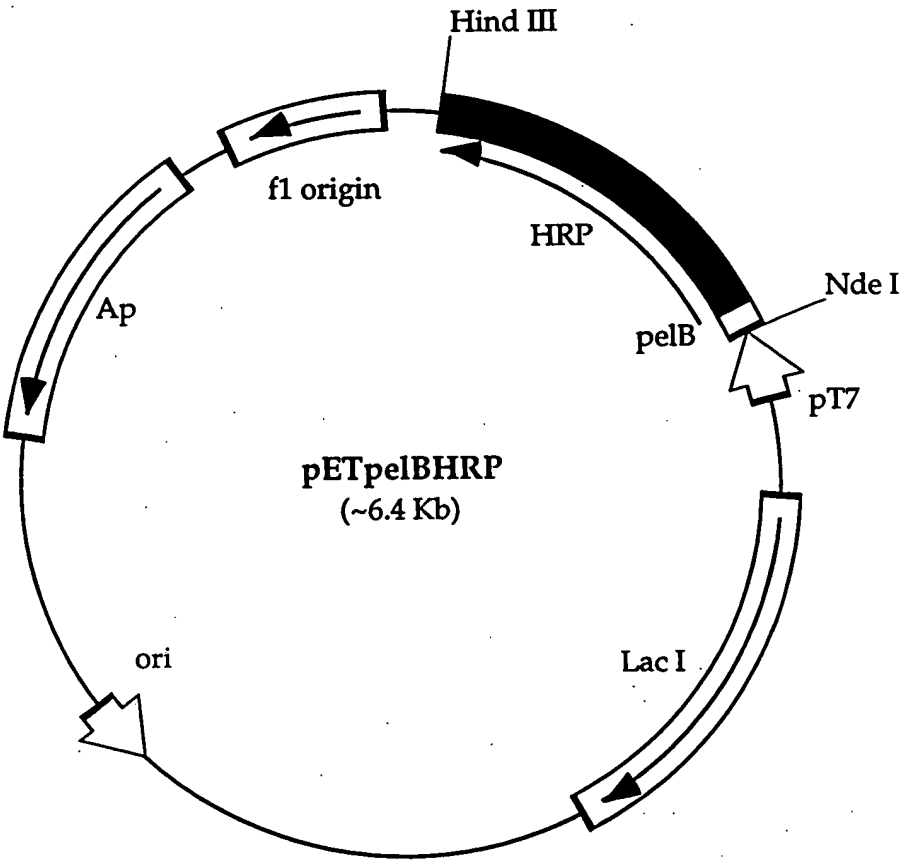

Hind III
f1 origin
HRP
Nde I
Ap
pelB
pT7
pETpelBHRP
(~6.4 Kb)
ori
Lac I

Fig. 21

ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA CCA GCC ATG GCC
Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala Met Ala

Fig. 22 Nucleotide and amino acid sequence of the pelb signal peptide.

[illegible]P mutant, HRP1A6, Coding Seq[illegible]e

(Muation, N255[illegible]AC --> GAC. The position of the [illegible]ting Met is 0)

```
        10        20        30        40        50        60
ATGCAGTTAACCCCTACATTCTACGACAATAGCTGTCCCAACGTGTCCAACATCGTTCGC
 M  Q  L  T  P  T  F  Y  D  N  S  C  P  N  V  S  N  I  V  R

        70        80        90       100       110       120
GACACAATCGTCAACGAGCTCAGATCCGATCCCAGGATCGCTGCTTCAATATTACGTCTG
 D  T  I  V  N  E  L  R  S  D  P  R  I  A  A  S  I  L  R  L

       130       140       150       160       170       180
CACTTCCATGACTGCTTCGTGAATGGTTGCGACGCTAGCATATTACTGGACAACACCACC
 H  F  H  D  C  F  V  N  G  C  D  A  S  I  L  L  D  N  T  T

       190       200       210       220       230       240
AGTTTCCGCACTGAAAAGGATGCATTCGGGAACGCTAACAGCGCCAGGGGCTTTCCAGTG
 S  F  R  T  E  K  D  A  F  G  N  A  N  S  A  R  G  F  P  V

       250       260       270       280       290       300
ATCGATCGCATGAAGGCTGCCGTTGAGTCAGCATGCCCACGAACAGTCAGTTGTGCAGAC
 I  D  R  M  K  A  A  V  E  S  A  C  P  R  T  V  S  C  A  D

       310       320       330       340       350       360
CTGCTGACTATAGCTGCGCAACAGAGCGTGACTCTTGCAGGCGGACCGTCCTGGAGAGTG
 L  L  T  I  A  A  Q  Q  S  V  T  L  A  G  G  P  S  W  R  V

       370       380       390       400       410       420
CCGCTCGGTCGACGTGACTCCCTACAGGCATTCCTAGATCTGGCCAACGCCAACTTGCCT
 P  L  G  R  R  D  S  L  Q  A  F  L  D  L  A  N  A  N  L  P

       430       440       450       460       470       480
GCTCCATTCTTCACCCTGCCCCAGCTGAAGGATAGCTTTAGAAACGTGGGTCTGAATCGC
 A  P  F  F  T  L  P  Q  L  K  D  S  F  R  N  V  G  L  N  R

       490       500       510       520       530       540
TCGAGTGACCTTGTGGCTCTGTCCGGAGGACACACATTTGGAAAGAACCAGTGTAGGTTC
 S  S  D  L  V  A  L  S  G  G  H  T  F  G  K  N  Q  C  R  F

       550       560       570       580       590       600
ATCATGGATAGGCTCTACAATTTCAGCAACACTGGGTTACCTGACCCCACGCTGAACACT
 I  M  D  R  L  Y  N  F  S  N  T  G  L  P  D  P  T  L  N  T

       610       620       630       640       650       660
ACGTATCTCCAGACACTGAGAGGCTTGTGCCCACTGAATGGCAACCTCAGTGCACTAGTG
 T  Y  L  Q  T  L  R  G  L  C  P  L  N  G  N  L  S  A  L  V

       670       680       690       700       710       720
GACTTTGATCTGCGGACCCCAACCATCTTCGATAACAAGTACTATGTGAATCTAGAGGAG
 D  F  D  L  R  T  P  T  I  F  D  N  K  Y  Y  V  N  L  E  E

       730       740       750       760       770       780
CAGAAAGGCCTGATACAGAGTGATCAAGAACTGTTTAGCAGTCCAGACGCCACTGACACC
 Q  K  G  L  I  Q  S  D  Q  E  L  F  S  S  P  D  A  T  D  T

       790       800       810       820       830       840
ATCCCACTGGTGAGAAGTTTTGCTAACTCTACTCAAACCTTCTTTAACGCCTTCGTGGAA
 I  P  L  V  R  S  F  A  N  S  T  Q  T  F  F  N  A  F  V  E

       850       860       870       880       890       900
GCCATGGACCGTATGGGTAACATTACCCCTCTGACGGGTACCCAAGGCCAGATTCGTCTG
 A  M  D  R  M  G  N  I  T  P  L  T  G  T  Q  G  Q  I  R  L

       910       920       930
AACTGCAGAGTGGTCAACAGCAACTCT
 N  C  R  V  V  N  S  N  S
```

Fig. 23

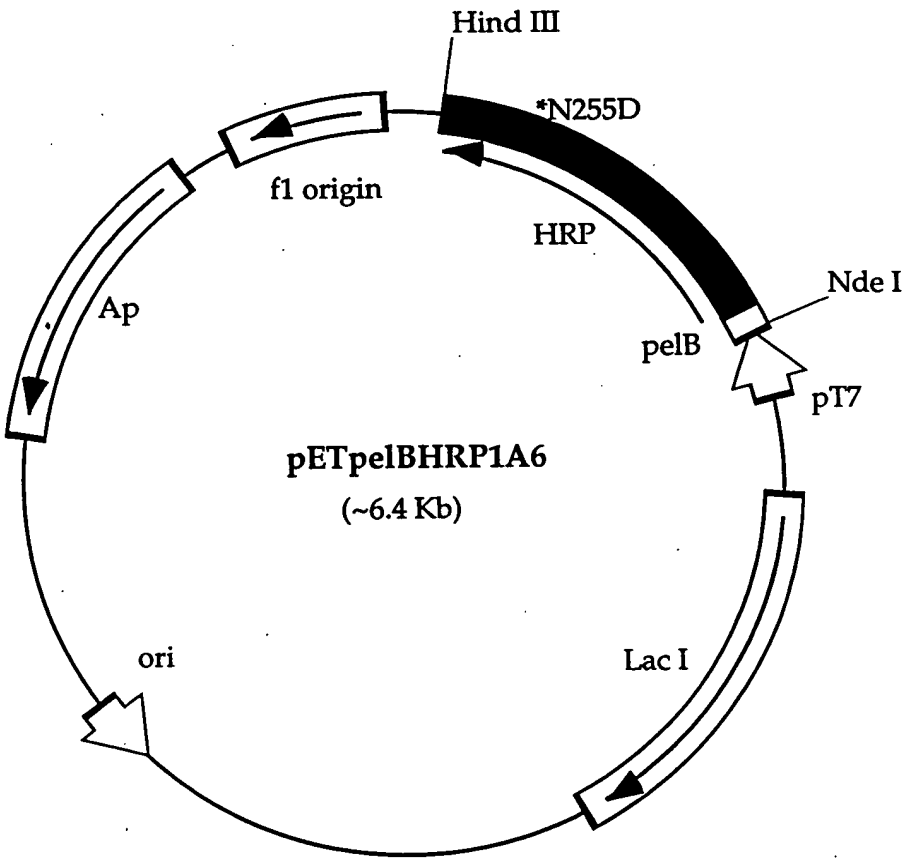

Hind III
*N255D
f1 origin
HRP
Nde I
Ap
pelB
pT7
pETpelBHRP1A6
(~6.4 Kb)
ori
Lac I

Fig. 24

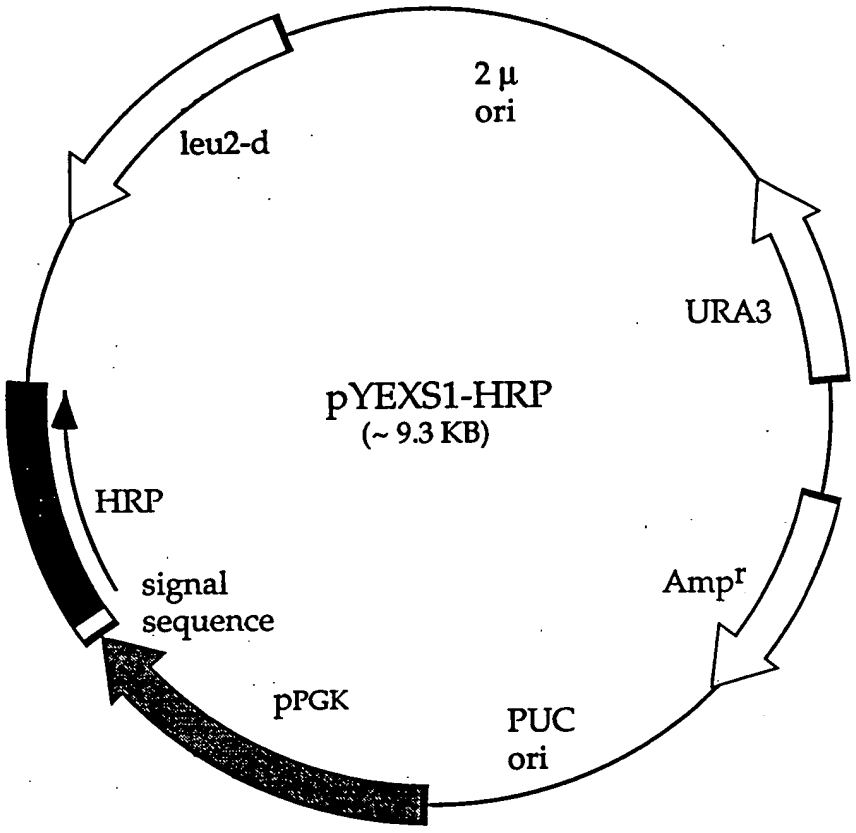

2 μ
ori
leu2-d
URA3
pYEXS1-HRP
(~ 9.3 KB)
HRP
signal
sequence
Amp^r
pPGK
PUC
ori

Fig. 25